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Changes in Salinity Preference of Threespine Stickleback over Development

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An honor's thesis by:

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ABSTRACT: Anadromous Threespine Stickleback, *Gasterosteus aculeatus*, migrate from freshwater to saltwater during their early development. The timing and extent of this migration could be influenced by salinity preference. The current study was done to observe if and when salinity preference changes during stickleback ontogeny. This study also looked at the effects of historical environmental salinity on salinity preference. The fish in this study, originating from an anadromous population from Cook Inlet, Alaska, were split into two groups and acclimated to a low (1ppt) and high salinity (30 ppt). After a period of acclimation, the fish were subjected to weekly salinity preference trials in gradient preference tanks or control non-gradient tanks for 9 consecutive weeks. This time span encompassed the time when migration would occur naturally. Individual fish were tested in both control and gradient conditions through repeated trials in a paired design. Each trial lasted for four hours, and position was measured every five minutes. Results indicated that fish were more active in control tanks as opposed to gradient tanks. This result conveys a preference, indicating that fish settle when finding a preferred salinity. We found that salinity preference changed with development and that fish experienced an intensifying drive for salt as they aged. This preference was anticipatory of migration as it occurred before the timing of natural migration. We also found that acclimation had no significant effects on salinity preference indicating that environmental salinity does not affect the intrinsic drive for salt. This study forces us to re-evaluate the importance of innate factors in juvenile fish migration and encourages us to look at salinity preference over development in other populations.

INTRODUCTION:

Migration is often a critical part of many species' life history. Dingle (1996) defined migratory behavior as "persistent and straightened out movement effected by the animal's own locomotory exertions or by its active embarkation on a vehicle. It depends on some temporary inhibition of station-keeping responses, but promotes their eventual disinhibition and recurrence". This definition is universal to animal movement patterns; however, animals exhibit differences in the mechanisms dictating their migratory behavior.

The question of "how do animals begin to migrate" has been thoroughly reviewed. A dichotomy of causation between environmental and innate factors seems to dominate scientific literature. Theories of migration often suggest that migration is a hereditary process (Woodbury 1941). When and where an animal moves may be dictated by genetics. In terms of innately controlled migrations, migrations are linked to development, as seen in the initial migration to the sea of certain fish species. Some studies show that environmental factors can motivate migration (e.g Audet et al. 1986). Baggerman (1960b) suggests migratory behavior occurs as a result of the combination of these two factors, with environmental cues acting as "releasers" for migratory behavior once fish are in a proper physiological state. This combination of both innate and environmental factors has been documented in certain species, such as barnacles. Barnacle larvae movement is directly impacted by innate factors as well sensory input (Baker 1982). Barnacles possess the capability to detect change in certain environmental conditions and have mechanisms to motivate the organism to attain their preferred environment (Baker 1982). In this scenario, it is essential that the barnacle possess the innate ability to be receptive to environmental conditions, leading to a combination of both factors contributing to movement.

During migration, organisms need to undergo changes whether innately controlled, or motivated by environmental cues to successfully migrate. The physiological changes that either prepare for or accompany the changing environmental conditions associated with migration could be reflected in preference for a particular environment.

Causation of migration in fish species has been significantly discussed within scientific literature. Migrations between fresh and saltwater are of great interest due to the drastic physiological and behavioral changes necessary when moving between fresh and salt water environments. This particular study looks at ontogenetic changes in preference in diadromous fish migration.

Diadromy

Diadromous fish migrate between freshwater and saltwater at some point in their lifetime (Myers 1949). This characteristic migratory behavior occurs in a wide variety of bony fishes (McDowall 1988). Despite occurring broadly phylogenetically, diadromous migrations are not always the same across species. Species display variation in the timing, reason for migration, and even pattern of migration. Based on differences among the reasons for migrating, migratory diadromous fish are categorized as anadromous, catadromous, or amphidromous. Although anadromy and catadromy were previously defined in the early 1700's, Myers created a formal definition for both these terms and, in addition, a new term amphidromy, in 1949 (McDowall 1992; Myers 1949). Anadromous fish, which will be the focus of this paper, hatch and develop in freshwater, migrate to the sea, and return to freshwater for spawning. This behavior is mostly known from studies on the Salmonidae, but it occurs in a number of families, including Petromyzontidae, Geotriidae, Mordacidae, Gasterostidae, Acipenseridae, Clupeidae, and Osmeridae (Shrimpton 2013). Catadromous fish behave oppositely in comparison to anadromous

fish by inhabiting fresh water as adults and returning to saltwater for spawning. Unlike the other two groups, amphidromous fish do not base their migrations around reproduction, but migrate between fresh and salt water based on other requirements.

For any type of migration, behavioral or physiological changes have to take place. Because diadromous fish, by definition, inhabit two different aquatic habitats during their lifetime, they are required to possess the capability to function in both environments, which may entail widespread changing of an organism's behavioral and physiological mechanisms. The following section looks at different anadromous species and the changes in they experience in relation to their migration.

Anadromous Species

Members of the Clupeidae are often anadromous. Zydlewski and Wilkie (2013) outline several characteristics of transitions between fresh and salt water in Clupeids. Allis Shad exhibit salt water tolerance during the beginning of migration, allowing for their entry into the sea (Leguen et al 2007). American Shad also experience a developmental shift leading to decreased hyperosmoregulatory ability in freshwater, corresponding to the migratory period (Zydlewski and Wilkie 2013; Zydlewski and McCormick 1997). Regarding environmental factors, migration of American Shad is correlated with decreasing temperature (Zydlewski et al 2003). In Shad migration, both innate and environmental factors play a role.

The most commonly discussed example of anadromous fish are members of the Salmonidae. Juvenile Salmon's migration from freshwater to saltwater revolves around a major ontogenetic change from parr to smolt. This shift includes changes in physiology, morphology, and behavior in preparation for migration to the marine environment (McCormick 2013).

Physically, a transition from being a banded parr to a thin streamlined smolt occurs sometime during the first few years of life, dependent on the species (National Research Council 2004; McCormick 2013). During smoltification, parr lose their parr marks, become silver, elongate, and experience an increased salinity tolerance (McCormick 1998). Smoltification is also specifically tied to development and will only occur at a certain size (McCormick 1998). Baggerman (1960) did an extensive study on salinity preference of juvenile Pacific Salmon. She found that salinity preference of four species of Salmon changed from fresh to salt water at the time of migration. In addition, by using radioiodine, she found higher thyroid activity in relation to migration, which is indicative of hormone production (some error). It was higher right before migration, continued to be high during migration, and declined at the end of migration (Baggerman 1960). This information shows that an ontogenetic change in thyroid activity may be responsible for initiating migratory behavior since it occurs previous to migration (Baggerman 1960). Other studies have confirmed Baggerman's observation of a change from a preference for fresh water to salt water preference in species of Salmon at the time of migration (McInerney 1959). Otto and McInerney (1970) showed a decrease in preference for freshwater as the time of migration approached. As for environmental cues, Baggerman (1960) found that photoperiod was correlated with changes in salinity preference.

An anadromous member of the Gasterosteidae, the Threespine Stickleback *(Gasterosteus aculeatus),* has been shown to undergo physiological and behavioral changes when migrating from freshwater to saltwater. This species consists of three populations including marine, freshwater, and anadromous (Bell and Foster 1994). Anadromous populations, one of which happens to be the test subject in this study, differ anatomically from their fresh water counterparts by being completely plated (Bell and Foster 1994). Physiologically, Threespine

Stickleback have been shown to withstand changes in environmental salinity (Campeau 1983). Current studies are underway looking at salinity tolerance of Threespine Stickleback at different ages. In relation to migratory behavior, salinity tolerance has been linked with seasonal variability (Lam and Hoar 1967). In addition to salinity tolerance, Threespine Stickleback also express variability in salinity preference. Baggerman (1957) tested stickleback preference under the influence of photoperiod, temperature and hormones. Other studies have confirmed her results that both environmental and physiological changes, such as photoperiod, temperature and hormones can induce a particular preference for either salt or fresh water (Audet et al 1985; Audet et al 1995; Audet et al 1986). Dave Fryxell (2012) reviewed salinity preference studies in Threespine Stickleback, and salinity testing devices, as well as describing his own testing salinity preference of marine, freshwater and anadromous populations. He found that anadromous fish generally preferred salt water when tested in salinity preference gradients (Fryxell 2012).

Salinity Tolerance and Salinity Preference

Anadromy requires an individual to perform different osmoregulatory processes in different salinity water. Fundamentally, fish inhabiting freshwater excrete water and absorb ions, while in salt water fish drink to maintain water concentration and expel ions (Edwards and Marshall 2013). The change in environmental salinity caused by migration requires a change in osmoregulatory mechanisms in order to function. This change in osmoregulatory ability can be represented by salinity tolerance. The development of tolerance for seawater can be correlated with early age development, size increase and gradual acclimation, or developed in preparation of migration through environmental cues (McCormick 1994). Salinity preference is a behavioral indication of physiological change needed for migration (Baggerman 1960). Preference mirrors the physiological changes occurring internally. We can assume that fish only exhibit a preference

for salinities they can tolerate. Therefore, we can use salinity preference as an indicator for migration, one which is used for orientation (McInerney 1959). Because ontogenetic changes have been observed relative to migration, this study looked at the development of anadromous juvenile Threespine Stickleback salinity preference and if it was controlled by innate mechanisms. In addition to control of ontogenetic changes, timing of these changes also impacts the drive for migration. If a preference occurs prior to migration, we could conclude that this preference is driving migratory behavior.

Objectives and Hypotheses

We are interested in looking at what drives migration in juvenile Threespine Stickleback. Using salinity preference as a mirror of physiological changes occurring in salinity tolerance, we can determine if preference is innately controlled or influenced by environmental factors. In natural conditions, in order for migration to be successful fish either have to possess innately controlled preparatory changes in preference, or they must be able to adapt to a new salinity forced upon them in a non-anticipatory fashion, or both. If we observe that a change in preference occurs in the absence of environmental cues, we can determine that this preference is innate and changes over ontogeny.

We intend to test the role of environmental factors on salinity preference by using two different acclimation treatments. Salinity preference can either change with age or be affected by acclimation, or both. For migration to be successfully, both acclimation and age can together cause a change preference, or just age can drive a change in preference. If we observe no change in preference with age, yet an acclimation effect, we can conclude that preference is not controlled by innate mechanisms, but that environmental history controls the observed salinity preference. If we observe the same change in salinity preference with age for the different

acclimations, we can determine that salinity preference is innately controlled and is intrinsic to the population, regardless of acclimation. If both acclimations experience consistent changes in preference with age in addition to an acclimation effect, we can conclude that preference is innately controlled and that environmental history influences salinity preference. And finally, if both groups experienced a different change in preference with age, we would conclude that salinity preference is innately controlled and that environmental history has an effect. If the change in preferences for each acclimation group merge together, we can determine that the group acclimated closest to the preferred salinity will be more successful at osmoregulating in that salinity and, therefore, will experience less change in preference with age.

Based on these possible outcomes, we hypothesized that relative to controls, stickleback will exhibit a preference shift towards higher salinity water as they age. We hypothesized that this preference will be controlled innately, however, there will be an acclimation effect, as seen in Dave Fryxell's (2012) study. We hypothesized that a preference will occur prior to the natural age of migration, indicating that salinity preference stimulates migratory behavior. Finally, we hypothesized that exposing fish to salinity gradients would stimulate searching behavior

METHODS:

Fish arrival

The Threepsine Stickleback used in this experiment were from an anadromous population in Rabbit Slough, Alaska. John Baker harvested and fertilized the embryos in Alaska and shipped them to Clark University. The embryos arrived on June 4th, 2013. Upon arrival, Clark students and staff divided and mixed the embryos, we transported the embryos to the University of Connecticut Atwater facilities that same day. We divided the embryos, now 48 hours old, into two groups: one to be acclimated to 1 ppt (Low group) and the other 30 ppt (High group). We

further split these acclimation groups into 15 petri dishes containing 25-30 embryos, with one additional 15 embryo dish per acclimation group. Using small paint brushes, we separated the embryos and removed non-viable eggs. We transferred the intact and separated eggs from Methylene Blue media to a new acclimation salinity created using Reverse Osmosis water and Instant Ocean (Aquarium Systems, Inc., Mentor, Ohio). Using a YSI (Yellow Springs Instruments, Yellow Spring, Ohio) sensor, we created these new salinities. We acclimated the Low group to 1 ppt immediately, while the High group began its acclimation at 6 ppt, increasing by 8 ppt daily. Through daily water changes, the High group reached 30 ppt after 4 days, while the Low group remained at 1ppt (Fig. 1).

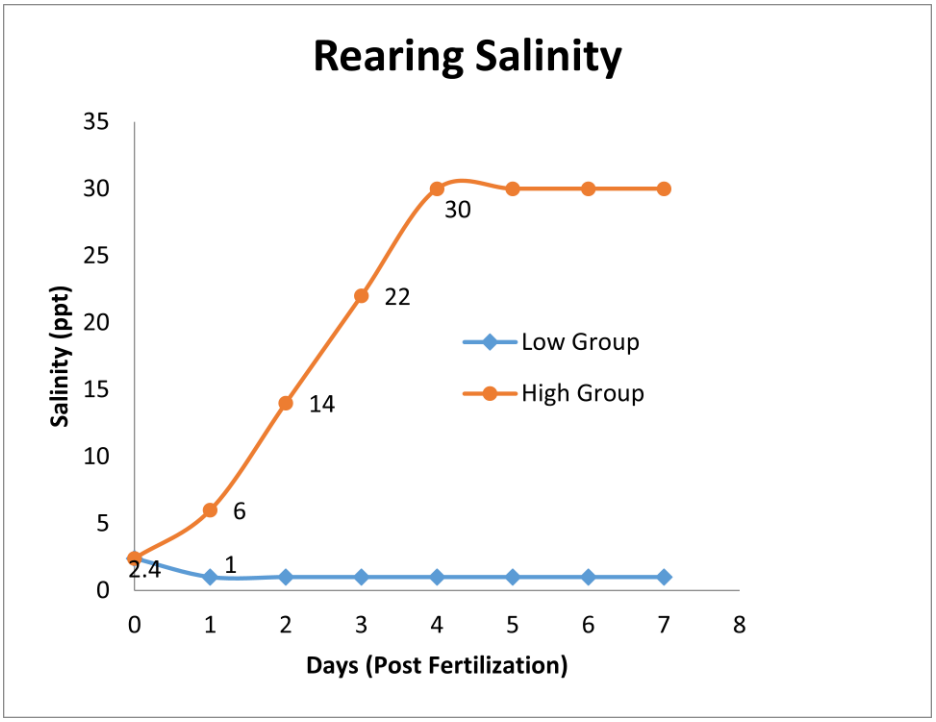

Figure 1. Graph of salinity acclimation of stickleback embryos.

Fish rearing

We followed a strategic timeline in order to maximize fish survival (Table 1a). We began by rearing embryos in Petri dishes for 10 days and performed daily water changes. Hatching began on June 9th, but did not occur in the majority of fish until June 10th. After hatching, we removed chorions along with mortalities. Hatching was similar between acclimations (Table 1b).

Table 1. a) Timeline for experiment b) Fish survival prior to salinity preference trials

a)

	Date
Fertilization	6/3/2013
Arrival at UConn- beginning of acclimation	6/4/2013
End of Acclimation	6/8/2013
Most Hatching	6/10/2013
First Successful Feeding	6/13/2013
Move to Jars	6/14/2013
Move to Tanks	6/18/2013
Salinity Preference Testing Begins in Small Preference Tanks	6/24/2013
Switch to Large Preference Tanks	7/15/2013
Environmental Enrichment Added	7/31/2013
Salinity Preference Testing Ends	8/20/2013

b)

	High Group (30 ppt)	Low Group (1 ppt)
Day 0	337	341
After Acclimation	325	323
After hatching	315	315
1st Successful Feeding	310	308
Transfer to Jars	307	305
Transfer to Tanks	241	295

Three days after hatching, we successfully fed fry brine shrimp nauplii (*Artemia salinas*) (Brine Shrimp Direct, Ogden, Utah) enriched with Selco (Brine Shrimp Direct, Ogden, Utah). After

successful feeding and 10 total days of rearing in Petri dishes, we transferred fish to quart jars, in which a water change occurred every other day. During this transition, high mortality occurred in some of the High group jars, which led to a large difference between the Low group and High group population sizes. Several High group jars were selected as unfit for experimentation and were placed in a scrap rearing tank not used for this experiment. Following a period of rearing in quart jars, we transferred the remaining healthy fish from quart jars to 10 gallon tanks. We combined Low group jars (1ppt) to compose three tanks of roughly 100 fish per tank, each maintained at 1 ppt for the rest of the experiment. Similarly, we combined High group jars (30ppt) into 2 tanks with roughly 100 fish per tank, each maintained at 30 ppt for the rest of the experiment. The large difference in population size between Low and High group tanks is a result of the removal of mortalities and unhealthy High group fish (Table 1b). Besides these removed unhealthy fish, we also removed some fish sporadically for specimen preservation as well as mRNA analysis.

Throughout the study, we reared fish in consistent conditions. We exposed fish to a light cycle of 14 hours light, 10 hours dark. Dimmers controlled the light cycle, allowing for an increase and decrease of available light similar to realistic environmental conditions. Throughout rearing, feeding technique changed. Feedings began consisting of brine shrimp nauplii and occurred three times daily. However we decreased nauplii feedings to twice daily at older ages. Later on in the study, we transitioned between food types, with fish eventually received a mixture of Golden Pearls (copepods) (Brine Shrimp Direct, Ogden, Utah) and nauplii daily and later one meal consisting of Golden Pearls and another consisting of nauplii. In addition to feeding, the rearing tanks also experienced some modification to better enhance fish survival. Rearing tanks included BioBricks® for collection of bacterial populations and shells to provide

calcium and other nutrients. In addition, we added environmental enrichment to the rearing tanks on July 31st, consisting of PVC pipe, false plants, and rocks.

Tank Construction

For salinity preference testing, we used preference tanks based on a design by Staaland (1969), later modified by Fivizzani and Spieler (1978). This device allows for the free movement of water between chambers while keeping a salinity gradient intact. We constructed the salinity preference tanks used in this experiment to include four chambers, each separated by upper and lower dividers. For this experiment, we constructed sixteen preference tanks, consisting of eight 1.8 liter and eight 18.9 liter tanks (Fig.2). The smaller 1.8 liter tanks consisted of Plexiglas melted together to form the tank shape. Some tanks required aquarium sealant on the edges to account for leaking. The larger tanks involved 5 gallon tanks with Plexiglas dividers attached using aquarium sealant. In addition, weather stripping was used to stop leaks between dividers.

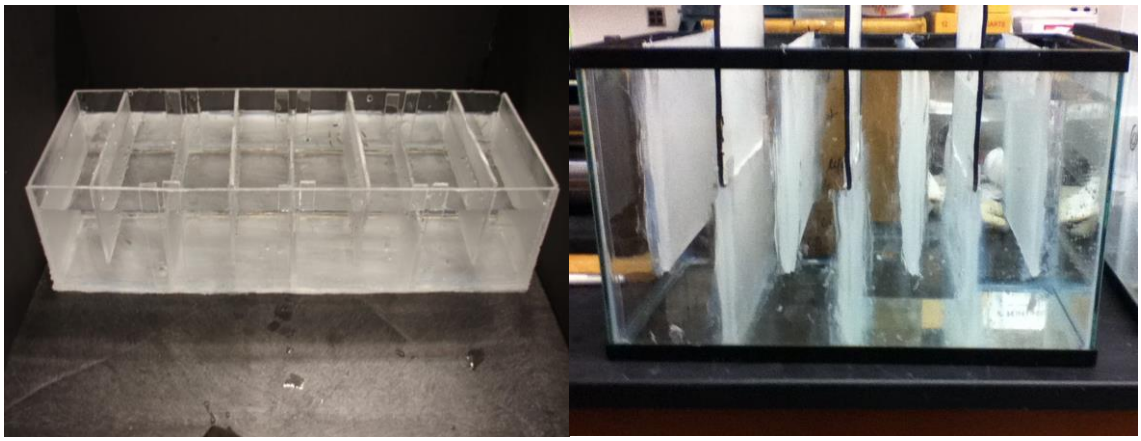

Figure 2. The left shows the 1.8 liter salinity preference tank and the right image shows the 18.9 liter salinity preference tank.

To prevent human interaction, we constructed two blinds to account for the different size tanks (Fig. 3). We used black tarp to created slits and flaps covered with mesh to minimize

observer interference with fish behavior. Experimental set up included two by four tanks on one table for the smaller tanks and two tables for the larger tanks. A blind surrounded the tables while being supported by PVC pipe and string. The blinds were taller than the observer and was easily disassembled. To prevent fish from seeing other fish in different tanks, we used foam poster board in between each tank.

Figure 3. Table set up and blind construction.

Experimental Design

We conducted salinity trials weekly starting on June 24th (Table 1a). Testing began when stickleback were 2 weeks post hatch. From ages 2 to 4 weeks post hatch, we used the small 1.8 liter tanks, and we used the larger 18.9 liter tanks from ages 5 to 10 weeks. Within each trial, we used a total of eight tanks, four used as preference gradient tanks and four used as controls (Fig. 4). Within the preference gradient tanks, two contained Low group acclimated fish and two contained High group acclimated fish. This was the same for the controls. To account for differences on the position of the testing table, we rotated the tanks before the start of each week. Each position was labeled A- H and each tank had a particular number assigned to it. This

allowed for the position of tanks to be rearranged to observe tank or position effects. In addition, we varied the position of the control and gradient tanks between trials.

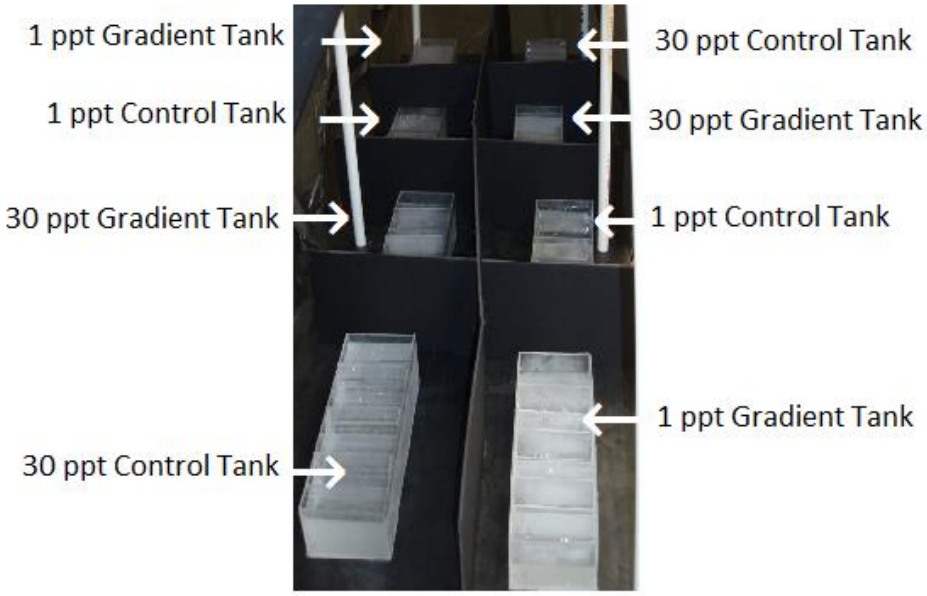

Figure 4. Example of experimental set up for each trial. Four tanks were used as gradient tanks, two for each acclimation, and four tanks were used as control tanks, two for each acclimation.

At the beginning of a preference trial, we filled each chamber in the modified Staaland tanks with a particular salinity to create a gradient. We filled the chambers with 0, 1, 3 and 30 ppt, ranging from freshwater to salt water (pH with distilled water: 6.64, 7.51, 8.00, and 8.19). The design of the gradient tank allows for intermixing of water but the gradient is maintained for both tank sizes (Fig. 5 and Table 2). During each trial, we varied the direction of the gradient between different tanks, proceeding either left to right or right to left.

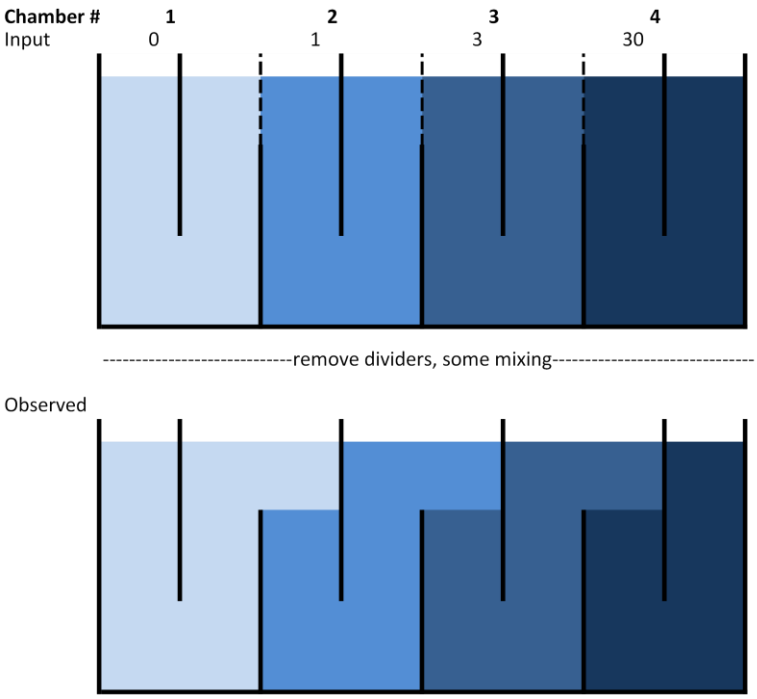

Figure 5. Changes in salinity in gradient tanks during a four hour trial.

Table 2. Change in salinity in gradient tanks over four hours. We measured salinity using a refractometer.

Chamber Number	1.8 liter tank	18.9 liter tank
1	0-1 ppt	0-0 ppt
2	1-2 ppt	0-2 ppt
3	3-7 ppt	2-8 ppt
4	16-30 ppt	8-30 ppt

Analysis of recorded salinities prior to and after trials indicated differences in the change in salinity occurring in each chamber (Table 3). Change was observed to be minimal and similar in lower salinity chambers in both tank sizes, allowing us to use the nominal salinity for calculations. However, we observed a greater changer in higher salinity chambers, especially in the 30 ppt chamber, for both acclimation groups.

Table 3. Change in salinity of chambers by tank size.

Nominal Salinity	Change in salinity of small tanks	Standard deviation of change in salinity of small tanks	Change in salinity of large tanks	Standard deviation of change in salinity of large tanks
0	0.52	0.67	0.24	0.40
1	1.2	1.1	0.32	0.46
3	5.6	2.5	2.6	1.0
30	-7.1	4.8	-7.1	3.2

During the trials, we created each salinity for each chamber by mixing RO water and Instant Ocean (Aquarium Systems, Inc., Mentor, Ohio). We used a YSI (Yellow Springs Instruments, Yellow Spring Ohio) and digital refractometer (Hanna Instruments, Woonsocket, Rhode Island) to make each salinity. We used the refractometer to measure and record the salinity of each chamber before and after each trial. However, due to error with the YSI, salinity measurements were slightly off during the experiments.

In comparison to experimental tanks, we filled control tanks with either 1ppt or 30 ppt water. After both experimental and controls were filled, we removed the dividers to allow for fish entry. During a trial, we removed four fish at random from each rearing tank and placed them in their corresponding control or gradient tank between chambers 2 and 3. After all fish entered the correct tank, the trial began. The observer recorded fish position through the blind every 5 minutes for four hours. When the trial ended we re-measured salinity of each chamber twice in different areas. We removed fish from the tanks and stored them in jars with their original acclimation overnight. We repeated the trails the next day (except for the first set of trials which occurred two days later) using the same stored overnight fish from the previous trial to test for preference among groups of fish. In the repeated trials, we tested fish in conditions

opposite to those they were tested in previously (Fig. 6). For example, we exposed fish that were previously exposed to gradient testing to control conditions, and vice versa.

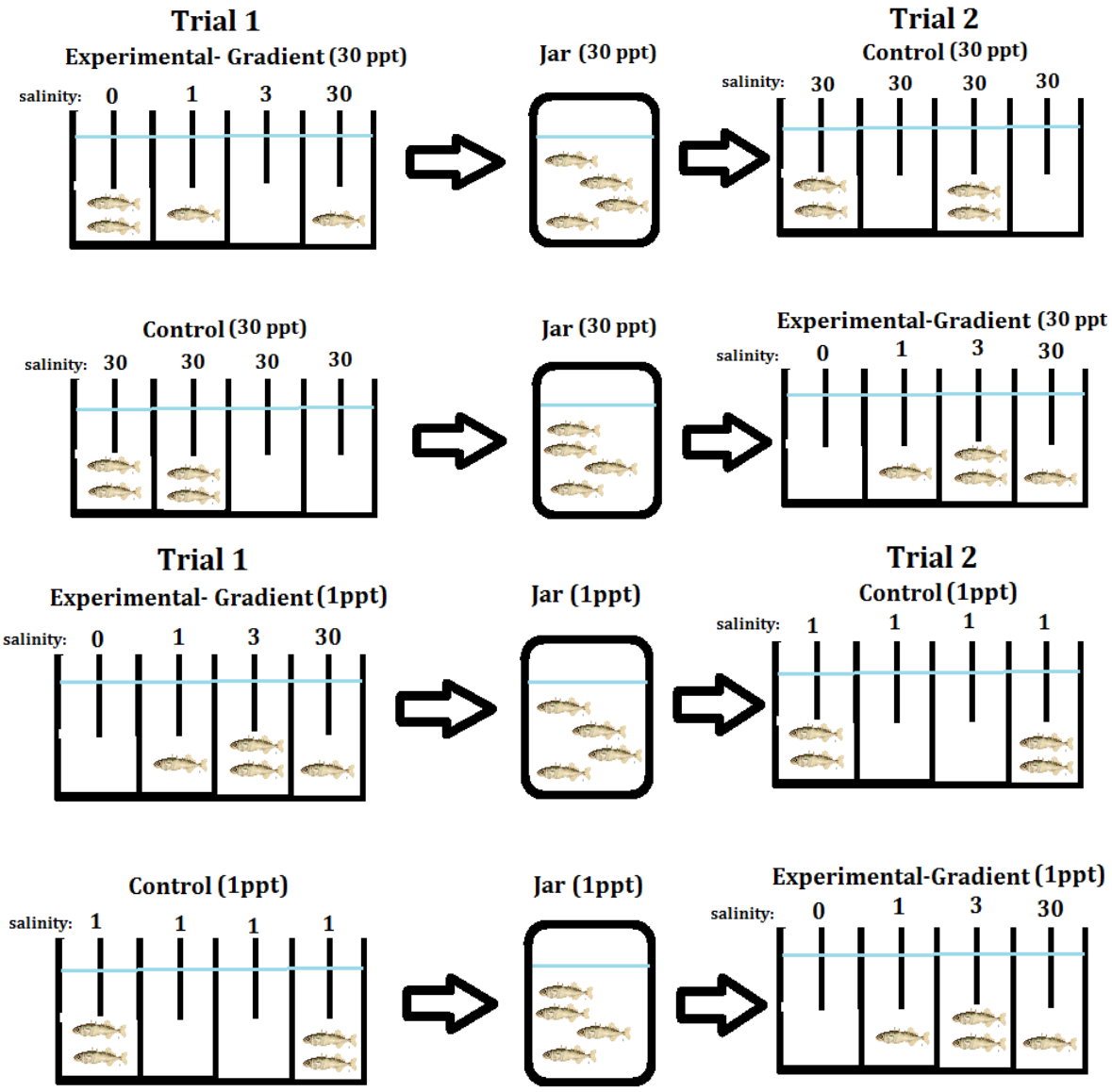

Figure 6. Schematic illustrating paired design. Fish in experimental tanks in trial 1 are exposed to control conditions in trial 2.

After the second trial occurred, fish were euthanized in MS-222. In total, trials occurred from week 2 to week 10, adding to 18 individual trials overall (Table 4). We used 16 fish per

each acclimation per trial, totaling to be 144 fish per acclimation, 288 fish for both acclimations over the whole trial.

Table 4. Timeline of salinity preference trials.

Salinity Preference Testing Week 1.1	6/24/2013
Week 1.2	6/26/2013
Salinity Preference Testing Week 2.1	7/1/2013
Week 2.2	7/2/2013
Salinity Preference Testing Week 3.1	7/8/2013
Week 3.2	7/9/2013
Salinity Preference Testing Week 4.1	7/15/2013
Week 4.2	7/16/2013
Salinity Preference Testing Week 5.1	7/22/2013
Week 5.2	7/23/2013
Salinity Preference Testing Week 6.1	7/29/2013
Week 6.2	7/30/2013
Salinity Preference Testing Week 7.1	8/5/2013
Week 7.2	8/6/2013
Salinity Preference Testing Week 8.1	8/12/2013
Week 8.2	8/13/2013
Salinity Preference Testing Week 9.1	8/19/2013
Week 9.2	8/20/2013

Data Analysis

Data analysis for this experiment was split into two parts: movement analysis and salinity preference. We conducted analysis for both using SAS (version 9.4, SAS Institute Inc., Cary, NC). Movement analysis consisted of comparing recorded fish position from consecutive time points to find the minimum amount of movement required to account for the difference in position between time points. This allowed us to compare mean movement of fish between controls and experimental, acclimation, and tank size. We found a spike of activity in the first hour that led us to omit that time period from the salinity preference analysis.

For the salinity analysis we decided to use the nominal salinity of each chamber. We decided this after finding the change in salinity in each chamber for each tank size. To conduct salinity preference analysis it was necessary to find an individual observation from each trial. To do this, we found the proportion of fish in each chamber by dividing the number of fish observations in each chamber by the total fish observations. To account for position effects, it was necessary to differentiate between control and gradient salinity preference analysis. For experimental gradient tanks, the preferred salinity was found by multiplying the proportion of fish in each chamber(P_{E_i}) by the nominal salinity of that chamber (S_{E_i}) (Equation 1). These values were summed to provide us with the preferred salinity for each gradient tank. This was averaged to provide us with the mean preferred salinity for that particular trial.

Equation 1.

$$\sum_{i=1}^{4} P_{E_i} \, x \, S_{E_i}$$

We conducted salinity preference analysis in the control tanks similarly to experimental analysis (Fig. 7). However, after finding that control fish distribution was symmetrical and that fish favored the end chambers, we symmetrized the proportion of fish observation data for covariate analysis. We calculated the proportion of fish observations in the end chambers (P_{C_a}) and the middle chambers (P_{C_b}) for each control tank to account for right and left sides of the tank (Equation 2). We found the proportion of fish in the end chambers by adding the proportion of

fish in chambers 1 (P_{C_1}) and 4 (P_{C_4}) and dividing that by 2. We found the proportion of fish observations in the middle chambers by adding the proportion of fish in chamber 2 (P_{C_2}) and 3 (P_{C_3}) and dividing that by 2.

Equation 2.

$$P_{C_a} = \frac{P_{C_1} + P_{C_4}}{2} \qquad P_{C_b} = \frac{P_{C_2} + P_{C_3}}{2}$$

After finding the proportion of fish observations in the middle and end chambers, we substituted these values into Equation 1 to find the preferred salinity of each tank. We multiplied the end (P_{C_a}) and middle observations (P_{C_b}) by the two corresponding nominal salinities ($S_{E_1} - S_{E_4}$) of the gradient tank (Equation 3). Because the control tanks consisted of only one constant salinity, this allowed us to find the expected preference (position preference) of the fish without an influence of salinity preference due to the presence of a salinity gradient.

Equation 3.

$$(P_{C_a} x S_{E_1}) + \left(P_{C_b} x S_{E_2}\right) + \left(P_{C_b} x S_{E_3}\right) + (P_{C_a} x S_{E_4})$$

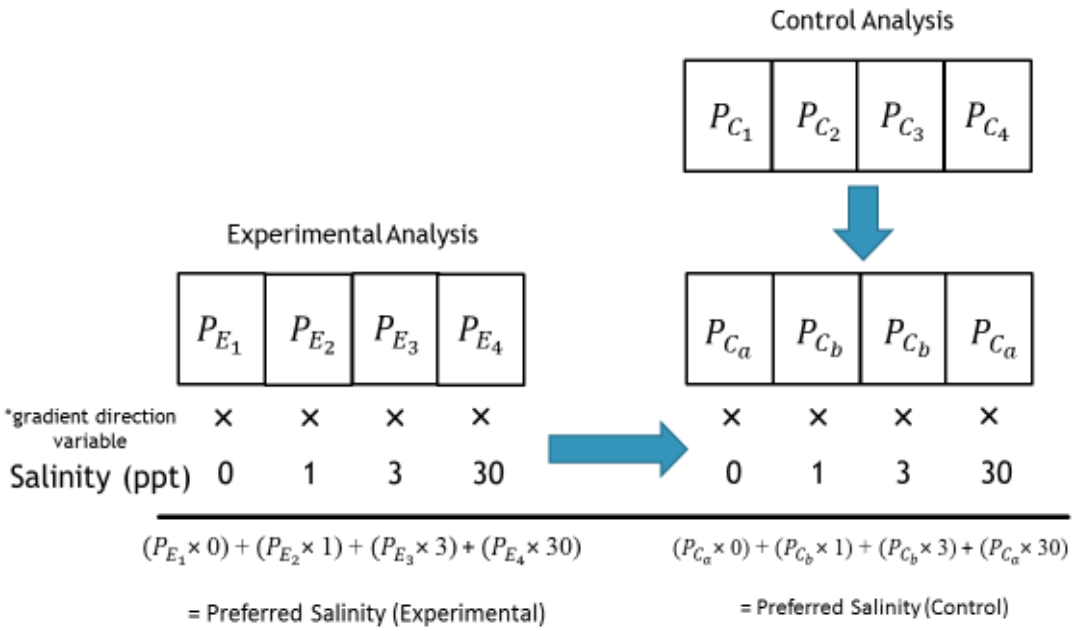

Figure 7: Schematic showing how preferred salinity was found.

After finding the preferred salinity for the control and gradient tanks, we found the mean preferred salinity for each condition for each age group. With this data, we ran an ANCOVA to test for variance in mean preferred salinity among control data (covariate), acclimation, age, and other interactions (Equation 4). We did not separate the analysis based on tank size due to the control encompassing any tank effects.

Equation 4.

$$Sal_{i,j,k} = \beta_1 Control_{i,j,k} + \gamma_2 acclimation_j + \beta_3 Age_i + interactions + \varepsilon_{i,j,k}$$

RESULTS:

Movement results indicated that fish moved more in control tanks, than in gradient tanks, in both tank sizes and acclimations. We also observed a large spike in activity with mean

movement peaking around 4 in the smaller 1.8 liter tanks during the first hour of the trials (Fig. a) his large spike was not observed in the large b) liter tanks. Because of this spike, we eliminated the first hour of observations in all trials in both tank sizes.

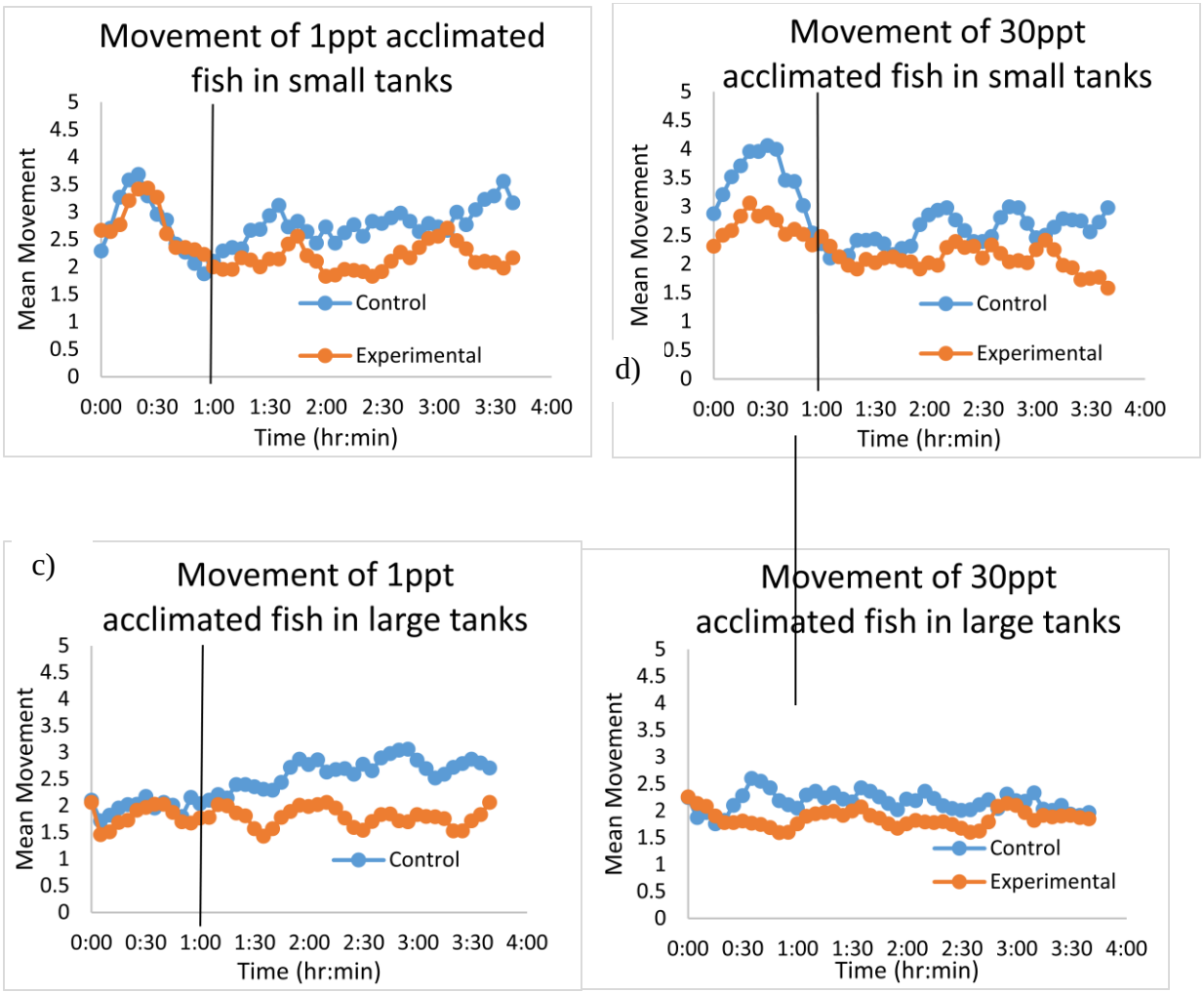

Figure 8. Average movement of fish (4 pt averages) by acclimation and tank size: a) 1 ppt, 1.8 liter tanks (small), b) 30 ppt, 1.8 liter tanks (small), c) 1ppt, 18.9 liter tanks (large), d) 30 ppt, 18.9 liter tanks (large).

Analysis of fish distribution showed a clear position preference. Fish clearly preferred the end chambers in control tanks for both tank sizes making the data appear symmetrical (Fig. 9).

The preference for the end chambers was more pronounced in the larger tanks than smaller tanks. Based on this result, we were able to symmetrize our control salinity preference analysis data.

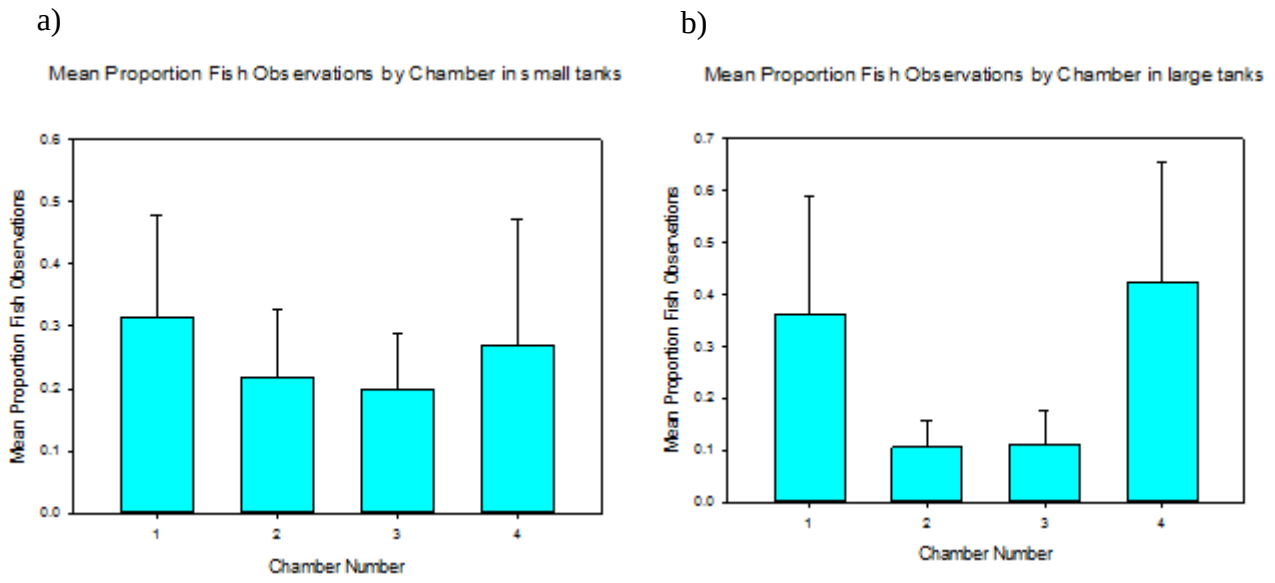

Figure 9. a) Control fish distribution by chamber in 1.8 liter (small) tanks b) Control fish distribution by chamber in 18.9 liter (large) tanks.

Week (age) and the covariate (control preference) had a significant effect on mean preferred salinity (Table 5). Acclimation had no significant effect on mean preferred salinity. There were no two or three way interactions in this data set and the mean preferred salinity of the trials was 17 ppt.

Table 5. ANCOVA results of covariate, acclimation and week on mean preferred salinity.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
covariate	1	147	147	5.7	0.02
Acclimation	1	0.55	0.55	0.02	0.9
Week	1	136	136	5.2	0.03

Preference for higher salinities increased with age in experimental fish (Fig. 10). As fish aged their drive for salt increased. We found that this increasing preference for higher salinity occurred in both acclimation groups.

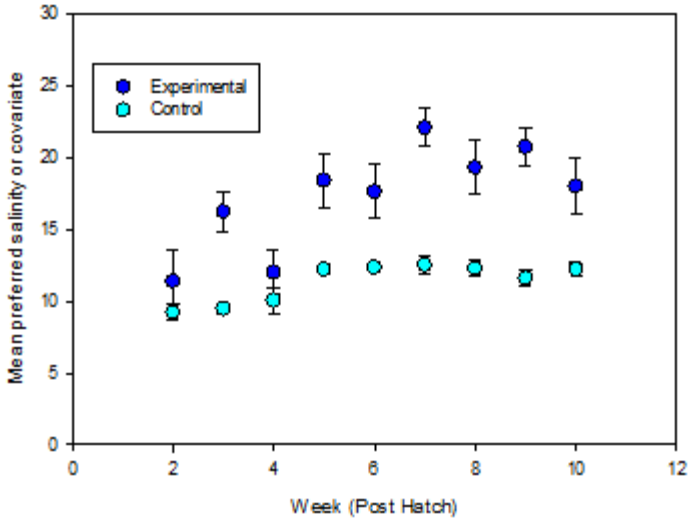

Figure 10. Mean preferred salinity in experimental tank or covariate estimated from position in control tank. Covariate represents expected salinity (preferred position) in absence of a salinity preference.

The covariate had a significant effect on mean preferred salinity. This indicates that position effects do influence the salinity preference data. We validated our results by subtracting the covariate salinity preference from the experimental salinity preference values to find the position corrected mean preferred salinity (Fig. 11). The position corrected salinity preference still increased with age.

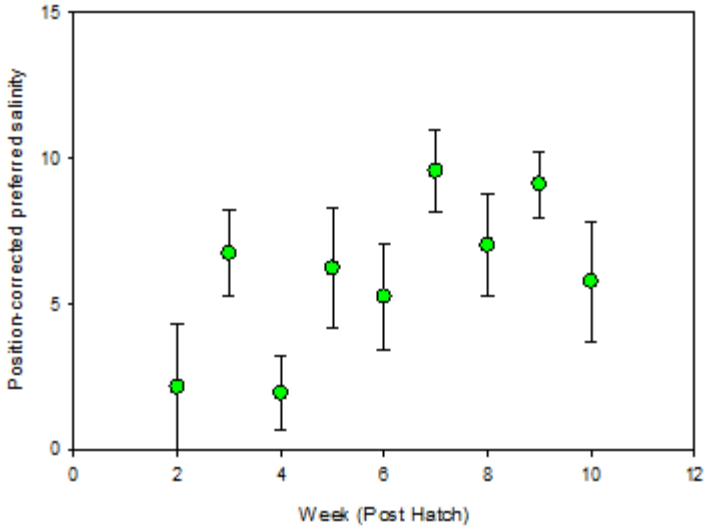

Figure 11. Position corrected salinity. Points represent the difference between the mean preferred salinity in experimental tanks and the covariate estimated from control tanks.

DISCUSSION:

Results indicated that control fish move more than experimental fish. Salinity preference analysis showed that fish experience an intensified drive for higher salinities as they age. This preference occurs regardless of acclimation. In addition, we found that a preference occurs before the natural age of migration.

Movement analysis

The results from the movement analysis indicate that fish in control tanks move more than fish in gradient tanks. This result contradicts our hypothesis by showing that a salinity gradient does not stimulate searching behavior, but instead promotes settling once the fish has found a preferred salinity. Fish have an innate position preference for the end chambers, as seen in the control fish distribution. When a gradient is present, fish settle in a chamber once finding a

preferred salinity. Because control fish are not exposed to a gradient, their distribution is dictated by a position preference for the end chambers. Control fish move more than experimental fish due to frequent switching ends of the tank.

The results from movement analysis also indicated a large spike in activity during the first hour in both control and experimental small tanks. This spike in movement could be explained as the fish's response to the stress of being introduced to a new environment. This pattern was not observed in larger tanks probably due to the increase in chamber size.

Salinity Preference Analysis

In natural conditions, can fish would not be exposed to a change in salinity similar to our gradient tank. However, we can use the gradient to provide a way for the fish to express preference and the internal changes that accompany it. In accordance with our hypothesis, juvenile stickleback expressed an ontogenetic change in their salinity preference. Mean preferred salinity increased as the fish aged for both acclimation groups. Therefore, this change in salinity preference reflects a change in salinity tolerance.

Regarding the timing of salinity preference, we observed that preference occurred in an anticipatory fashion. The timing of migration of juvenile Threespine Stickleback has been observed to be between 4 and 8 weeks of age (Pers. comm. John Baker). Because a preference for higher salinities occurred prior to this time point, we can conclude that this salinity preference is anticipatory of migration. In her 1960 review, Baggerman (1960b) mentions the idea of a "migration disposition". This means that a fish will undergo different physiological changes in preparation for migration. However, migration will only occur once "released" by external factors. Baggerman (1957, 1960) has proven that salinity preference is related to

changes in physiological processes. In confirmation of Baggerman's (1960b) "migration disposition" theory, salinity preference could act as a causal factor for this disposition. Otto and McInerney (1970) conducted a similar study that tested salinity preference of salmon through their development. They found that a loss in preference for freshwater, as seen when approaching smoltification, is indicative of an increase in "migratory capacity" as opposed to "migratory disposition" (Otto and McInerney 1970). In both cases, salinity preference is representative of a change in tolerance.

In this study we found that a preference change occurred without external cues prior to the timing of migration. Environmental cues, such as photoperiod and temperature (e.g. Baggerman 1957, 1960; Audet et al 1986), have been shown to induce a salinity preference change. Baggerman has mentioned that environmental cues might act as "releasers" for the already present drive for higher salt (Baggerman 1960b). In this study, the environmental cue of acclimation treatment did not influence salinity preference.

Although we've concluded that salinity preference occurs prior to natural migration, why does juvenile stickleback salinity preference increase over a migratory season and not initially start at preference for oceanic conditions? A gradual increase in the preference for higher salinity water could drive movement into brackish water, and later saltwater. As indicated by McInerney (1964), salinity preference could drive a fish into saltier water, where once they encounter salt water, they experience further changes in preference which cause them to prefer oceanic salt water. Also, a gradual increase in salinity preference could parallel the ontogenetic changes occurring in osmoregulatory competence. Fish may only experience a preference for water they are able to comfortably osmoregulate in. An increase in salinity preference could also indicate the imperativeness of migration. As the season progresses, salinity preference will increase in

order to stimulate movement into salt water. This increasing drive towards salt water could occur so the fish do not miss the migration season. The stronger the preference for salt, the more intense the drive to migrate to saltwater from freshwater.

This study also found that the environmental history of organisms had no effect on preference. Acclimation conditions did not affect mean preferred salinity in either group. This is significant because the drive for salt is intrinsic to the organism and overcame any environmental change. This indicates that anadromous fish born in different salinity water will all experience the same drive for salt water that dictates their movement into the ocean. We expected fish acclimated to 30 ppt would experience a smaller change in salinity preference with age because they could already osmoregulate in the preferred salinity. We expected 1ppt acclimated fish to experience a larger change in salinity preference with age due to their lack of osmoregulatory ability for higher salinities. We found that both acclimations experienced the same driver for salt, indicating that salinity preference is innately controlled.

Our results differed from Dave Fryxell's (2012) study, which found that acclimation conditions did slightly impact salinity preference. One explanation for this could be the age at which acclimation occurred. In this study embryos were acclimated to the correct salinity within the first few days. In Fryxell's study, fish were not acclimated until 3 weeks post hatch, right prior to the timing of natural migration. This difference in rearing technique could have attributed to the difference in acclimation effects. When transferred to a new salinity, fish have to utilize different mechanisms to osmoregulate. In my study, fish underwent this transition as embryos and had a long developmental period after acclimation to prepare for migration. Because Fryxell's acclimation period occurred so close to migration, fish may have already begun undergoing physiological changes in osmoregulatory abilities. For example, fish that were

acclimated to freshwater may have already begun experiencing a preference for salt at the time of acclimation. This development could be connected with changing osmoregulatory mechanism. However, this late transfer may have delayed the development of a preference for salt by forcing the fish to osmoregulate at a lower salinity. Because this study acclimated fish so early, there was no delay in osmoregulatory ability.

Within trials, we often observed all four fish collectively in a chamber. It is important to note that even if the fish preferred a certain chamber they could act territorial and aggressive. In this scenario, the salinity preference would not be adequately shown. We did not observe any antagonistic behavior between fish, most likely due to their age of testing.

For future studies, we recommend extending salinity preference testing past the time of migration to see if salinity preference continues to increase with development in Threespine Stickleback. It would be interesting to see if preference plateaus or continues to increase to that of pure seawater. Based on observations from Figure 10, we predict that preference will plateau before reaching 30 ppt. In addition, it would be useful to test other anadromous populations of Threespine Stickleback to see if this increasing preference for salt is universal. Based on our above data, we can interpret that due to migration within a chamber to a preferred salinity, migration in the wild would occur. Future studies could extend the length of the salinity testing apparatus to conclude that migration would occur in the preferred direction. Finally, a more broad study could test the theory of "migratory disposition" versus "migratory capacity" in other anadromous species.

This study found that anadromous Threespine Stickleback experience an intensifying drive for higher salinities. This increase in salinity preference may be responsible for driving juvenile migrations into the ocean, as it occurs prior to the timing of natural migration. Due to a

lack of acclimation effects we can conclude that this drive for seawater is innate to these fish, occurring in groups exposed to different environmental histories. Based on this research, we confirm that salinity preference may be one of the factors causing anadromous fish to migrate.

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References

Audet, C. FitzGerald, G.J., and Guderley, H. 1986. Environmental control of salinity preferences in four sympatric species of sticklebacks: *Gasterosteus aculeatus, Gasterosteus wheatlandi, Pungitius pungitius* and *Apeltes quadracus*. Journal of Fish Biology 28: 725-739

Audet, C. FitzGerald, G. J., and H. Guderley. (1985). Salinity Preferences of Four Sympatric Species of Sticklebacks (Pisces: Gasterosteidae) during Their Reproductive Season. Copeia 1:209-213.

Audet, C., Fitzgerald, G.J. and H. Guderley. (1995). Prolactin and Cortisol Control of Salinity Preferences in *Gasterosteus aculeatus* and *Apeltes quadracus*. Behaviour 93:36-55

Baggerman, B. 1957. An experimental study on the timing of breeding and migration in the three-spined stickleback (*Gasterosteus aculeatus* L.). Arch. Neerl. Zool. 12:105-318.

Baggerman, B. 1960. Salinity Preference, Thyroid Activity and the Seaward Migration of Four Species of Pacific Salmon (*Oncorhynchus*). Journal of the Fisheries Research Board of Canada 17:295-322.

Baggerman, B. 1960b. Factors in the diadromous migration of fish. Symposia of the Zoological Society of London 1:33-60.

Baker, R. Robin. 1982. Migration paths through time and space. London: Hodder and Stoughton.

Campeau, S .. H. Guderly, and G. Ftizgerald. 1984. Salinity tolerances and preferences of fry of two species of sympatric sticklebacks: possible mechanisms of habitat segregation. Can. J. Zool. 62: 1048-1051.

Dingle, Hugh. 1996. Migration: The Biology of Life on the Move. New York, New York: Oxford University Press.

Edwards, S. L. and W. S. Marshall. 2013. Principles and patterns of osmoregulation and euryhalinity. McCormick, Stephen D., Farrell, Anthony P. and Colin J. Brauner (Eds.), *Eurhyhaline* Fishes Waltham, MA: Academic Press.

Fivizzani, A. J. and R. E. Spieler. 1978. Modified Staaland device with automatic recording techniques for determining salinity preference in fishes. J. Fish. Res. Board Can. 35: 910 912.

Fryxell, D. 2012 "Salinity Preference of Alaskan Threespine Stickleback: Test for Divergence in Halotaxis between Ancestral and Landlocked Populations" *Honors Scholar Thesis.*

Lam, T.J. and Hoar, W.S. 1967. Seasonal effects of prolactin on freshwater osmoregulation of the marine form (trachurus) of the stickleback *Gasterosteus aculeatus*. Canadian Journal of Zoology 45:509-516.

Leguen, I. et al. 2007. Development of hypoosmoregulatory ability in allis shad (*Alosa alosa*). Journal of Fisheries Biology 70:630-637.

McCormick, Stephen D. et al. 1998. Movement, migration, and smolting of Atlantic salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Science 55: 77-92.

McCormick, Stephen D. 1994. Ontogeny and Evolution of Salinity Tolerance in Anadromous Salmonids: Hormones and Heterochrony. Coastal and Estuarine Research Federation 17: 26-33.

McCormick, Stephen D. 2013. Smolt Physiology and Endocrinology. McCormick, Stephen D., Farrell, Anthony P. and Colin J. Brauner (Eds.), *Eurhyhaline* Fishes Waltham, MA: Academic Press.

McDowall, R.M. 1988. Diadromy in Fishes: Migrations between freshwater and marine environments. London: Croom-Helm.

McDowall, R.M. 1992. Origins and Definitions of Terminology. American Society of Ichthyologists and Herpetologists. 1:248-251.

McInerney, J.E. 1959. Salinity Preference- An orientation mechanism in salmon migration. Thesis- University of British Columbia

McInerney, J.E. 1964. Salinity preference: an orientation mechanism in salmon migration. J. Fish. Res. Bd. Canada 21:995-1017.

Myers, G.S. (1949). Usage of Anadromous, Catadromous and Allied Terms for Migratory Fishes. Copeia 2: 89-97

National Research Council. Atlantic Salmon in Maine. 2004. Washington, D.C.: National Academies Press

Shrimpton, Mark J. 2013. Seawater to freshwater transitions in diadromous fish. McCormick, Stephen D., Farrell, Anthony P. and Colin J. Brauner (Eds.), *Eurhyhaline* Fishes Waltham, MA: Academic Press.

Staaland, H., 1969. A device for the study of salinity preference in mobile marine animals. Comparative Biochemistry and Physiology 29, 853-857.

Otto, R.G. and McInerney, J.E. 1970. Development of Salinity Preference in Pre-Smolt Coho Salmon, *Oncorhynchus kisutch*. Journal of the Fisheries Research Board of Canada 27:793-800.

Woodbury, Angus M. 1941. Animal Migration: Periodic-Response Theory. The Auk 58:463-505.

Zydlewski, J. and McCormick, S. D. 1997. The loss of hyperosmoregulatory ability in migrating juvenile American shad, *Alosa sapidissima*. Canadian Journal of Fisheries and Aquatic Science 54: 2377-2387.

Zydlewski, J., McCormick, S.D. and Kunkel, J. G. 2003. Late migration and seawater entry is physiologically disadvantageous for American shad juveniles. Journal of Fisheries Biology 63:1521-1537.

Zydlewski, J. and M. Wilkie. 2013. Freshwater to seawater transitions in migratory fishes. McCormick, Stephen D., Farrell, Anthony P. and Colin J. Brauner (Eds.), *Eurhyhaline* Fishes Waltham, MA: Academic Press.